

(19) World Intellectual Property Organization
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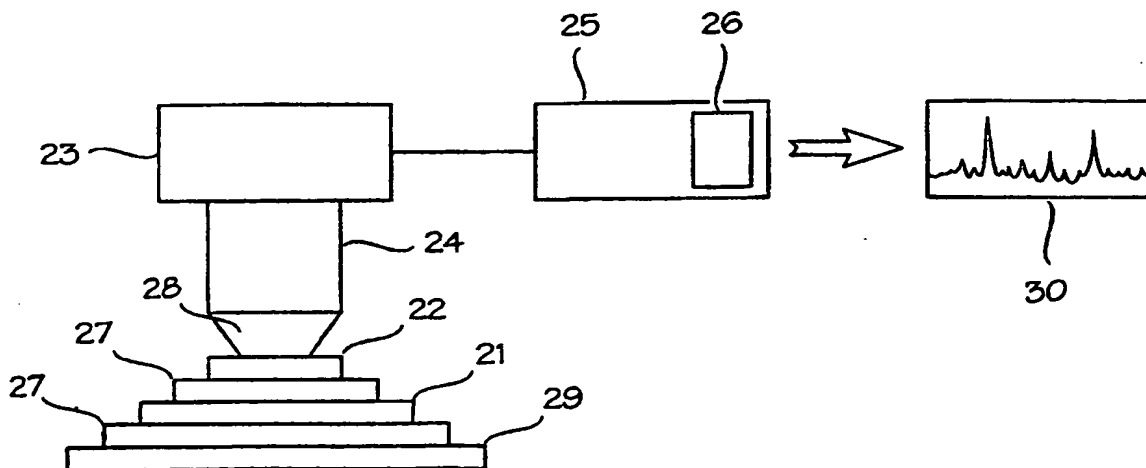
(43) International Publication Date
25 May 2001 (25.05.2001)

PCT

(10) International Publication Number
WO 01/36949 A1

- (51) International Patent Classification⁷: **G01N 21/65**
- (21) International Application Number: **PCT/FI00/00981**
- (22) International Filing Date:
10 November 2000 (10.11.2000)
- (25) Filing Language: **Finnish**
- (26) Publication Language: **English**
- (30) Priority Data:
19992439 12 November 1999 (12.11.1999) **FI**
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- (81) Designated States (national): AE, AG, AL, AM, AT, AT
(utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA,
CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility
model), DK, DK (utility model), DM, DZ, EE, EE (utility
model), ES, FI, FI (utility model), GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility
model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG,
MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD,
SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT,
TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— With international search report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANALYSING A SAMPLE IN RAMAN SPECTROSCOPY



(57) Abstract: This invention relates to a method of analysing a sample (21) in Raman spectroscopy, the method comprising tuning the molecules of the sample (21) with monochromatic light, collecting and analysing the light that has Raman scattered from the sample (21) with a spectrometer (25). The method comprises using a cover slip (22) below an objective (24), filling the space between the objective and the cover slip (22) with a first immersion liquid (28) and the space between the cover slip (22) and the sample (21) surface with a second immersion liquid (27), and selecting said second immersion liquid (27) so that the refractive index of the second immersion liquid (27) is substantially the same as the refractive index of the sample (21) to be analysed.

ANALYSING A SAMPLE IN RAMAN SPECTROSCOPY

This invention relates to a method of analysing a sample in Raman spectroscopy, the method comprising tuning the molecules of the sample with monochromatic light, and collecting and analysing the light that has Raman scattered from the sample with a spectrometer. The invention also relates to a measurement arrangement for performing Raman spectroscopy, the measurement arrangement comprising a sample, an immersion objective, a spectrometer and a source of monochromatic light.

The invention is related to sample analysis which requires high accuracy, and particularly to performing mapping measurements in the depth direction. It is known that sample analysis of this kind is performed in Raman spectroscopy. The analysis provides information on the vibration of molecules, which can be utilized for identifying the sample and finding out its chemical composition. By means of a microscope the analysis can be restricted to a very small volume of the sample. The sample molecules are tuned with monochromatic light. Part of this light Raman scatters from the sample to be analysed so that the light wavelength changes. The Raman scattered light is collected and analysed with a spectrometer. The intensity of this Raman scattered light as a function of frequency is called the Raman spectrum of the sample. The spectrum provides both quantitative and qualitative results, and the spectrum can be compared with reference lists and digital databases.

Immersion objectives are used in the prior art to improve the resolution. This is achieved using a substance with a refractive index higher than that of air together with an immersion objective. Immersion objectives are generally used in light microscopy, whereas in Raman spectroscopy they are used only in some special applications. For example, when biological samples are analysed with the Raman spectroscopy, water immersion objectives are sometimes used, mainly to prevent excessive heating of the sample.

In the measurement arrangements described above problems are caused by reflection of the tuning light and the light that has Raman scattered from the sample on the sample surface, scattered light and an imaging error. These phenomena are caused by the fact that the beams of light are subjected to great differences in refractive index, and thus they prevent the tuning light from penetrating into the sample and focusing on it. Scattered light is random reflection that occurs on the sample surface and is caused by variations in the refractive index on the surface which is rough due to pores and different

particle forms. The scattered light weakens the resolution in the x,y plane. The imaging error, i.e. sample aberration, results from refraction of light on the sample surface. The beams with different angles of incidence refract differently and the common focal point is lost. This weakens the depth resolution and collection efficiency may decrease even by half.

5 An object of the present invention is to solve the above-mentioned problems and to provide a method for performing Raman spectroscopy more efficiently and accurately than before. This object is achieved with a method of the invention which is characterized by filling a space between an objective and the sample with an immersion liquid, and selecting said immersion liquid so that the refractive index of the immersion liquid is substantially the same as the refractive index of the sample to be analysed. A second method of the invention is characterized by using a cover slip below the objective, filling the space between the objective and the cover slip with a first immersion liquid and the space between the cover slip and the sample with a second immersion liquid, and selecting said second immersion liquid so that the refractive index of the second immersion liquid is substantially the same as the refractive index of the sample to be analysed.

15 The invention also relates to a measurement arrangement for performing Raman spectroscopy which can be utilized in the application of the method according to the invention. The measurement arrangement of the invention is characterized in that the measurement arrangement also comprises an immersion liquid for filling a space between the objective and the sample, the refractive index of the immersion liquid being substantially the same as that of the sample. A second preferred embodiment of the invention is characterized in that the measurement arrangement also comprises a cover slip, a first immersion liquid between the immersion objective and the cover slip and a second immersion liquid between the cover slip and the sample, the refractive index of the second immersion liquid being substantially the same as the refractive index of the sample to be analysed.

20 The invention is based on the idea of eliminating the great differences in refractive index between the objective and the sample which cause light refraction and reflection, which are disadvantageous to the analysis. When the space between the objective and the sample is filled with an immersion liquid which is selected so that its refractive index is very close to that of the sample, light can travel the whole distance from the objective inside the

sample and the collected Raman scattering can travel back to the objective without great variations in the refractive index. This provides several advantages. The most important advantages are the following: more light penetrates into the sample as the sample surface becomes smooth and variations in the refractive index are eliminated when the immersion liquid fills the pores on the sample surface, and light focuses better on one point since there will be no imaging error. In that case the collection efficiency and depth resolution of the Raman scattered light improve. A numerical aperture, i.e. an NA value, can be used as the gauge of collection efficiency. The value is determined according to the following formula

$$NA = n \sin \alpha$$

where n is the refractive index of the medium and α is the angle of incidence at which a beam hits the sample. In practice, the maximum value of the numerical aperture is 0.95 when a dry objective is used, but considerably higher NA values are achieved with an immersion objective. If the refractive index of the sample is 1.5, the NA value of the dry objective will be 0.63 according to the above-mentioned formula. Thanks to the increased collection efficiency, the measurement times can be reduced even by half, and thus the sample does not heat up too much. An additional advantage is that the immersion liquid cools down the sample, which enables use of a tuning light with a higher power without damaging the sample.

In a preferred embodiment of the method according to the invention immersion liquid is added both above and below the sample. In that case no air can be left below the sample, which might cause above-mentioned problems resulting from discontinuities in the refractive index.

In a second preferred embodiment of the method according to the invention a liquid which is substantially not Raman active is selected as the immersion liquid to be used on the sample surface. This means that immersion liquids with a strong Raman spectrum are eliminated because the spectrum might overlap with the spectrum of the sample to be analysed and thus disturb the analysis.

The preferred embodiments of the method and measuring arrangement of the invention are disclosed in the dependent claims.

In the following, the invention will be described by way of example

with reference to the accompanying drawings, in which

Figure 1 is a flow chart illustrating a method according to the invention, and

5 Figures 2A and 2B illustrate a measurement arrangement according to the invention.

Figure 1 is a flow chart illustrating a method according to the invention. It is assumed, by way of example, that a paper coating is analysed by measuring it in the depth direction by means of Raman spectroscopy. A microscope is used for restricting the analysis to a very small sample volume. An immersion objective, a cover slip and two different immersion liquids are used
10 in the measurement.

In step 1A a cover slip is placed below the objective, and the sample to be analysed, e.g. coated paper, is placed on the slide below the cover slip. The refractive index n_N of the paper coating used as an example is about
15 1.55. It consists of pigment, e.g. calcium carbonate with a refractive index of approximately 1.56, and of a binder, e.g. styrene butadiene latex with a refractive index of approximately 1.55. In addition, the paper coating may totally or partly consist of kaolin pigment with a refractive index of approximately 1.55. In step 1B the space between the objective and the cover slip is filled with a
20 first immersion liquid, which is immersion oil with a refractive index n_1 of approximately 1.5. This immersion oil has been selected so that its refractive index is very close to that of the cover slip, which is also about 1.5. In practice, the manufacturer of the immersion objective also supplies the immersion oil together with the objective. The manufacturer of the objective may also state
25 what kind of cover slip should be used with the immersion objective. The cover slip is a separate part, and in special cases the immersion objective can also be used without the cover slip. In step 1C a liquid with a refractive index as close to that of the sample as possible is selected as a second immersion liquid. In this example a liquid with a refractive index close to 1.55 is thus suitable for use as the second immersion liquid. Silicon oil with a refractive index
30 n_2 of about 1.5 is selected as the second immersion liquid. This second immersion liquid is used for filling the space between the cover slip and the sample and the space between the sample and the slide. The actual analysis starts in step 1D. The molecules of the sample are tuned with monochromatic light, i.e. with laser. In this example the light wavelength is 785 nm, but other wavelengths could also be used. The Raman spectrum of the second immer-

sion liquid should not be too strong or overlap with the Raman spectrum of the sample. The silicon oil and the paper coating function together rather well in this respect, too. In step 1E the light that has Raman scattered from the sample is collected and analysed with a spectrometer. As a result of the analysis, the spectrometer provides a Raman spectrum which describes the intensity of the Raman scattered light in relation to the frequency. Streaks in the Raman spectrum indicate chemical properties of the sample at the measured depth. In step 1F the distance between the objective and the sample is changed e.g. by increasing the sample level by 1 μm and by performing the analysis at this depth. The distance can be changed repeatedly, in which case distribution of a certain substance into different depths of the sample can be found out by following changes in the Raman spectrum. The sample can also be moved in the x,y plane. In step 1G deconvolution is performed. This means that a concentration profile is calculated for the sample on the basis of the response function of the Raman instrument and the measurement result, i.e. intensity of the Raman function. This profile allows more accurate analysis of the results. In the formula that expresses the function of the depth of Raman intensity

$$i(z) = \int_{-\infty}^{\infty} f(z-x)s(x)dx$$

$f(z)$ is the response function of the Raman instrument, which has the form $f(z-x)$ when it is integrated over the sample thickness, and $s(z)$ is the concentration profile, which is unknown at this stage. According to the convolution theorem, the following holds true

$$I(z) = F(z)S(z),$$

where the capitals represent Fourier transformations of the corresponding functions. The function $s(z)$ that represents the concentration profile is calculated from the formula

$$S(z) = \frac{I(z)}{F(z)},$$

from which the function $s(z)$ representing the concentration is finally obtained

by inverse Fourier transformation.

Figure 2A illustrates a measurement arrangement according to the invention. The measurement arrangement shown comprises a microscope 23, an immersion objective 24, and a Raman spectrometer 25 for performing the analysis, a laser 26 being used as the source of tuned light in the spectrometer. The measurement arrangement also comprises a slide 29 onto which a sample 21 is placed, a cover slip 22, and a first 28 and a second 27 immersion liquid. The figure also shows a Raman spectrum 30 which is obtained as a result of the spectrometer analysis. The streaks in the spectrum provide information on the chemical properties of the sample. The layers of a stack formed between the slide 29 and the objective 24 are pressed tightly together before the analysis so that there would be no air causing discontinuities in the refractive index between the layers. This stack can be moved in the vertical direction to adjust the focus on different depths of the sample 21. For this purpose the measurement arrangement may comprise means for adjusting the distance between the objective 24 and the sample 21. The objective 24 may include an adjustment based on the piezoelectric phenomenon, for example, for adjusting the distance by means of a computer. The adjustment margin can be 100 μm , for example. The distance adjustment may also be mechanical and provided in the microscope. Adjustment in the x,y plane can be performed by means of micrometer screws. When the distance between the objective 24 and the sample 21 is adjusted, the thickness of the immersion liquid layer 28 between the objective 24 and the cover slip 22 changes.

The measurement arrangement shown in Figure 2A can be used for analysing e.g. a paper coating up to the depth of 30 μm . In the case of a dry sample, i.e. when the light travels in the air between the objective and the sample, only a depth of about 4 μm can be achieved. The Raman spectrometer 25 may be e.g. a confocal Raman spectrometer in which a pinhole structure improves positional resolution, i.e. restricts the access of photons generated by Raman scattering outside the focal point to the detector. For example, the immersion oil 28 which is supplied by the manufacturer of the objective and has a refractive index very close to that of the cover slip 22 can be used together with the immersion objective 24. The second immersion liquid 27, the selection of which is central to this invention, can be e.g. silicon oil, glycerine, xylene, toluene, liquid teflon or water, depending on the refractive index of the sample to be used, the Raman spectrum and the wavelength and fluores-

cence of the tuning light. A liquid which changes the composition of the sample 21 cannot be selected as the second immersion liquid 27. Thus e.g. dissolving substances cannot be used. The immersion oil 28 supplied by the manufacturer of the immersion objective 24 is also suitable for use as the second immersion liquid 27, provided that the refractive index of the sample 21 is close to the refractive index of the immersion oil 28 but the Raman spectrum of the sample does not overlap with that of the immersion oil 28. Thanks to good positional resolution, the Raman scattering of the first immersion liquid 28 and the cover slip 22 does not have a disadvantageous effect on the Raman spectrum measured from the focal point. Typical samples 21 that can be analysed by the measurement arrangement of the invention include multi-layer polymer films, paper coatings and light-scattering coatings and paints.

Figure 2B illustrates another preferred embodiment of the measurement arrangement according to the invention, in which no cover slip is used with the objective 24 of the microscope 23, but the space between the objective 24 and the sample 21 as well as the space between the sample 21 and the slide 29 are filled with one immersion liquid 27'. Otherwise the embodiment of Figure 2B corresponds to that shown in Figure 2A.

The same principles apply to the selection of the immersion liquid 27' as to the selection of the second immersion liquid in Figure 2A. The most important requirement is that the refractive index of the immersion liquid 27' should be as close to the refractive index of the sample 21 as possible. The Raman spectrum of the immersion liquid 27' should not be too strong or overlap with the Raman spectrum of the sample 21. Furthermore, the immersion liquid 27' should not cause any changes in the properties of the sample 21.

It is to be understood that the above description and the drawings related thereto are only intended to illustrate the present invention. Those skilled in the art will recognize that many modifications and variations may be made without departing from the spirit and scope of the present invention defined in the appended claims.

CLAIMS

1. A method of analysing a sample (21) in Raman spectroscopy, the method comprising

5 tuning the molecules of the sample (21) with monochromatic light,
 collecting and analysing the light that has Raman scattered from the
sample (21) with a spectrometer (25), **characterized by**
 filling the space between an objective (24) and the sample (21) sur-
face with an immersion liquid (27'), and

10 selecting said immersion liquid (27') so that the refractive index of
the immersion liquid (27') is substantially the same as the refractive index of
the sample (21) to be analysed.

2. A method of analysing a sample (21) in Raman spectroscopy, the method comprising

15 tuning the molecules of the sample (21) with monochromatic light,
 collecting and analysing the light that has Raman scattered from the
sample (21) with a spectrometer (25), **characterized by**
 using a cover slip (22) below an objective (24),

20 filling the space between the objective (24) and the cover slip (22)
with a first immersion liquid (28) and the space between the cover slip (22) and
the sample (21) surface with a second immersion liquid (27), and

 selecting said second immersion liquid (27) so that the refractive in-
dex of the second immersion liquid (27) is substantially the same as the refrac-
tive index of the sample (21) to be analysed.

25 3. A method according to claim 1 or 2, **characterized** in that
immersion liquid (27, 27') is also added below the sample (21).

 4. A method according to any one of claims 1 to 3, **character-
ized** in that a liquid which is substantially not Raman active is selected as
the immersion liquid (27, 27') to be used on the sample surface.

30 5. A method according to any one of claims 2 to 4, **character-
ized** in that the same liquid is selected as the first (28) and the second (27)
immersion liquid.

 6. A method according to any one of claims 1 to 5, **character-
ized** in that the sample (21) is analysed at different depths by adjusting the
distance between the objective (24) and the sample (21).

7. A measurement arrangement for performing Raman spectroscopy, the measurement arrangement comprising a sample (21), an immersion objective (24), a spectrometer (25) and a source (26) of monochromatic light, **characterized** in that the measurement arrangement also comprises

5 an immersion liquid (27') for filling the space between the objective (24) and the sample (21), the refractive index of the immersion liquid being substantially the same as the refractive index of the sample (21).

8. A measurement arrangement for performing Raman spectroscopy, the measurement arrangement comprising a sample (21), an immersion objective (24), a spectrometer (25) and a source (26) of monochromatic light, **characterized** in that the measurement arrangement also comprises

10 a cover slip (22), a first immersion liquid (28) between the immersion objective (24) and the cover slip (22) and a second immersion liquid (27) between the cover slip (22) and the sample (21), the refractive index of the
15 second immersion liquid (27) being substantially the same as the refractive index of the sample (21) to be analysed.

9. A measurement arrangement according to claim 8, **characterized** in that the same liquid is used as the first (28) and the second (27) immersion liquid.

20 10. A measurement arrangement according to any one of claims 7 to 9, **characterized** in that the immersion liquid (27, 27') is also used below the sample (21).

11. A measurement arrangement according to any one of claims 7 to 10, **characterized** in that the measurement arrangement comprises
25 means for adjusting the distance between the objective (24) and the sample (21) for analysing the sample at different depths.

12. A measurement arrangement according to any one of claims 7 to 11, **characterized** in that the sample (21) to be analysed is polymer film and the immersion liquid (27, 27') is silicon oil.

30 13. A measurement arrangement according to any one of claims 7 to 12, **characterized** in that the sample (21) to be analysed is paper coating and the immersion liquid (27, 27') is silicon oil.

1/2

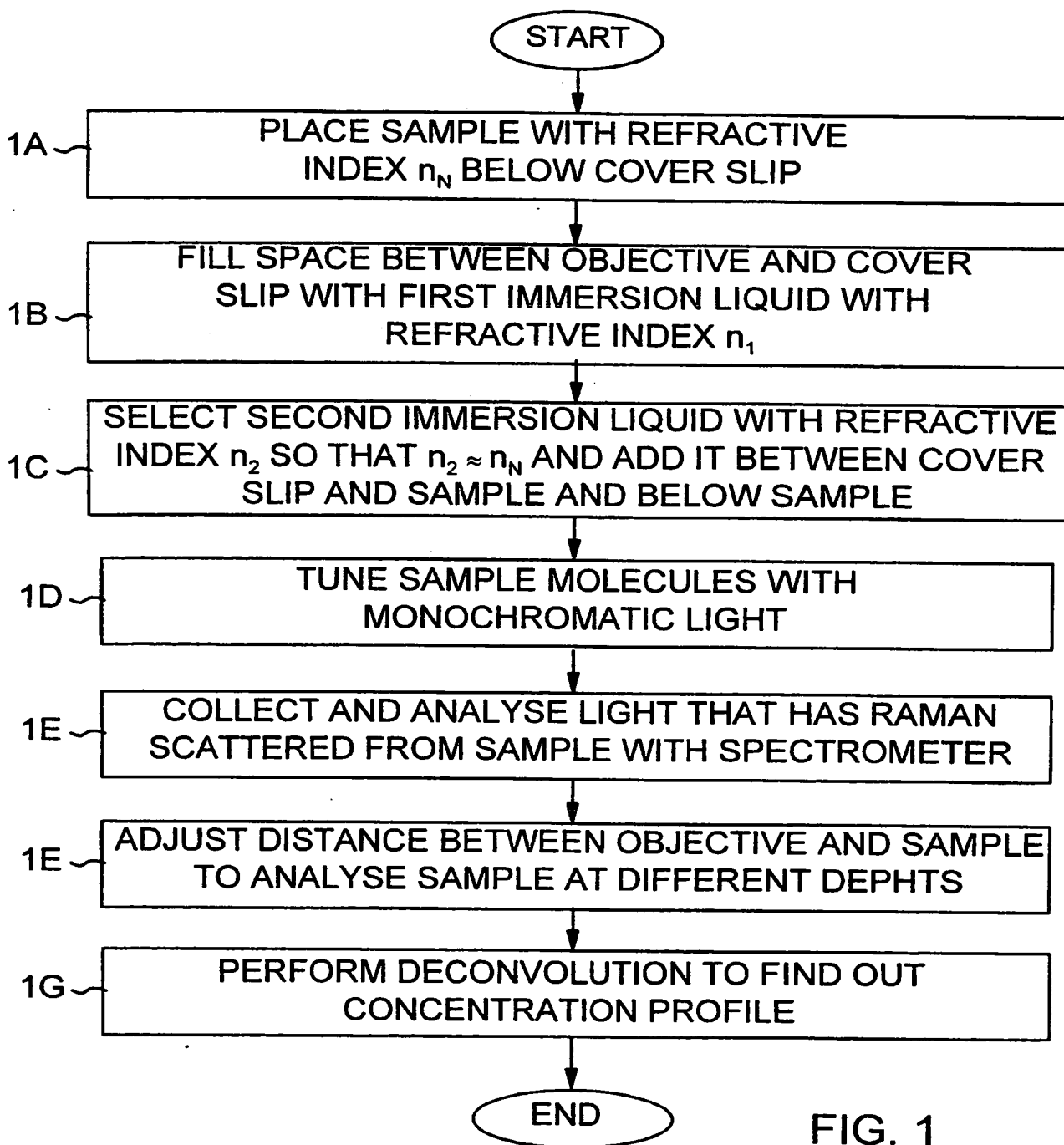


FIG. 1

2/2

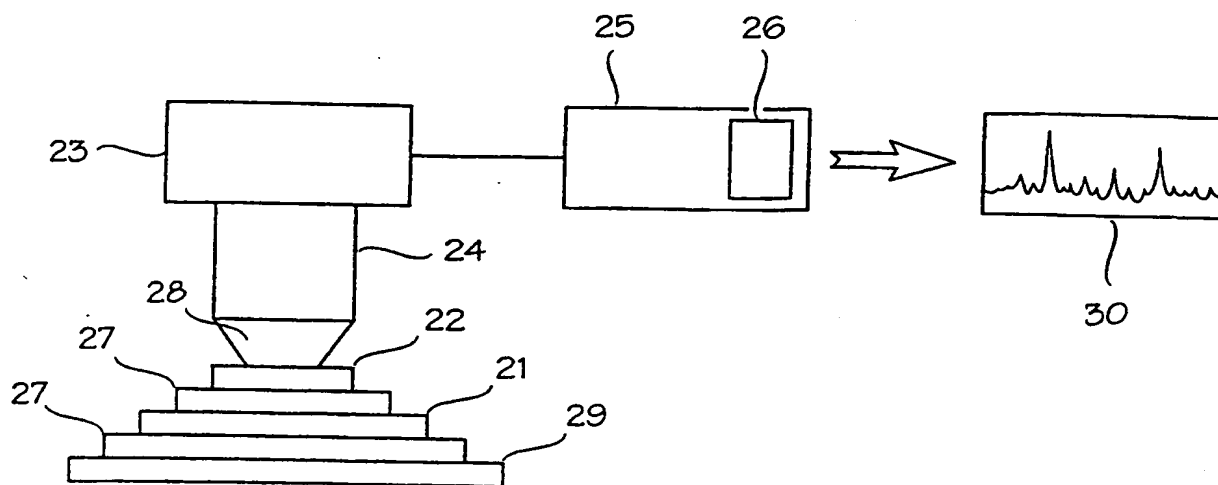


FIG. 2A

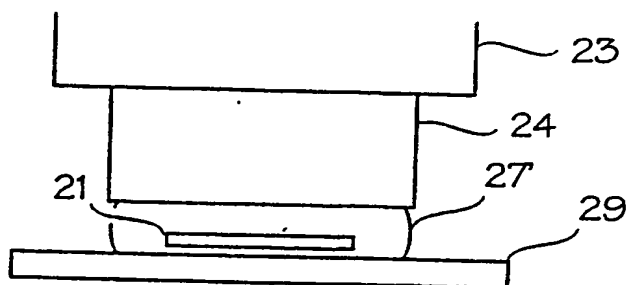


FIG. 2B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00981

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 21/65

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0503236 A2 (INTERNATIONAL BUSINESS MACHINES CORPORATION), 16 Sept 1992 (16.09.92), page 5, line 45 - line 54; page 7, line 6 - line 11, figure 2b, abstract	1,3-7,10-11
A	abstract --	2,8-9,12-13
Y	US 4411525 A (T.OGAWA), 25 October 1983 (25.10.83), column 6, line 39 - line 59, abstract --	1,3-7,10-11
A	US 4714345 A (B.SCHRADER), 22 December 1987 (22.12.87), claims 5-8, abstract -- -----	1-13

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 February 2001

Date of mailing of the international search report

25 -02- 2001

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/FI 00/00981

Patent document cited in search report				Publication date		Patent family member(s)	Publication date
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US	4411525	A	25/10/83	DE	2904787	A,C	09/08/79
				DE	7903488	U	06/09/79
				JP	54109488	A	28/08/79
US	4714345	A	22/12/87	DE	3424108	A,C	09/01/86
				FR	2566903	A,B	03/01/86
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				JP	61020841	A	29/01/86